LESSON 25:
BIOCHEMICAL PRODUCTS OF INTEREST – PART II
OTHER PRODUCTS

Objective

Cultured plant cells are known to produce biochemicals of interest since 1950's, but initially the yields were very low. Refined culture systems have improved the biochemical yields considerably, and over half a dozen cell cultures produce 2g/l or more of the biochemical. These biochemicals have impressive biological activities which are used in various agricultural drugs. Some compounds are used as food additives as flavours, fragrances, colours, etc. In this Lesson we will deal with such compounds and also certain other miscellaneous biochemicals of interest.

Agricultural Drugs

Plant Virus Inhibitors

A large number of chemically synthesized compounds and natural molecules have been examined for their inhibitory effects on plant viruses and some of them have potent activity as protectors against virus infection.

In order to screen high producing cultured cells of plant viral inhibitors, a variety of callus extracts were examined for the inhibitory activity towards tobacco mosaic virus (TMV) infection using a tobacco disc method by Misawa et al. of Kyowa Hakko in Japan. Among these extracts Phytolacca americana callus was selected as the most potent producer of the inhibitor. The level of the inhibitor accumulated in the suspension cultured cells and reached maximum level on the 9th day of culture using MS medium containing 1 mg/l 2,4-D. The cell suspension of P. americana was homogenized and the supernatant was diluted up to 100 times with water. The diluted solution was found to inhibit TMV infection on tobacco and tomato plants significantly.

Further studies in the same laboratory used Agrostemma githago, a more potent producer of the plant virus inhibitor. The growth of suspension cultured cells of A. githago was somewhat faster than P. americana.

Active principles of P. americana and A. githago were isolated with Column-lite chromatography and electrofocusing. At least four basic proteins were obtained from P. americana cells whose molecular weights were $1.10X10^4$ to $3.15X10^4$ Among them the highest molecular weight component contained sugars in the molecule. On the other hand, only one basic protein was isolated as the principle from A. githago culture and its molecular weight was $2.5X10^4$. The proteins obtained from P. americana have been widely investigated because of their activities to HIV.

Ikeda et al. of Japan Tobacco Inc. also screened various plants and selected Mirabilis jalapa as a producer of an anti-plant virus protein. The callus was induced from leaves of M. jalapa and its suspension cultured cells established were found to accumulate the protein intracellularly. Optimization of the production and the cell growth as well as selection of high producing cell lines were conducted extensively. One of the lines produced 95 mg/L of the protein in the optimized medium based on MS medium on the 7th day of the cultivation. Its molecular weight was 24 KD and the amino acid sequence was determined which had 24% homology with a ribosome-inactivating protein, ricin D-A chain.

Food Additives

Pigments

Shikonin Compounds

Shikonin and its derivatives such as acetyl shikonin and isobutyl shikonin accumulated in roots of Lithospermum erythrorhizon are reddish purple pigments and have been used in traditional dyeing. The plant has also been used as a herbal medicine. Because of a shortage of this plant, Fujita et al. at Mitsui Petrochemical in Japan investigated mass cultivation of L. erythrorhizon cells to produce shikonin compounds. Using the cell line established by Tabata's laboratory of Kyoto University, they optimized its culture conditions extensively to increase the level of the products using flasks and various types of fermentors including a rotating cylindrical fermentor designed by Tanaka et al. of Tsukuba University.

Fujita et al. found that L. erythrorhizon produced shikonins in White's medium but the cell growth was poor in the same medium. On the other hand, Linsmair-Skoog's (LS) medium was recognized to support the growth but not shikonin production. Therefore, they used a two-stage culture for mass production of shikonin compounds. Namely, to proliferate the cells, LS medium was used at the first stage of the fermentation, and then the cells were transferred into White's medium for production of shikonin compounds. In order to improve the yields further, optimization of components in both media was carried out extensively, and MG-5 and MG-9 media were established,

Since most cultured cells in liquid and solid media occur as aggregates, selection of high-producing cell lines from the aggregated cells is not effective and labour-intensive. The Mitsui group prepared protoplasts from the cultured cells with appropriate enzymes and selected high shikonin compounds-producing protoplasts using a cell sorter. The selected protoplasts were generated to cell lines and cultivated in suspension. From 48 cell lines, they obtained a cell line having 1.8 fold the productivity of the parent line. The cell line showed stable production of shikonin compounds.

To produce the compounds more efficiently, the same group attempted to employ a high-cell density culture process in the second stage of the two-stage cultures. By feeding the nutrients into M-9 medium, the level of cell mass increased and that of the compounds produced increase twice as much as without feeding. Shikonin and its derivatives are being manufactured

commercially by the company. A major application of the pigment is for lipsticks.

Shimomura et al. established a hairy root culture of L. erythrorhizon with Agrobacterium rhizogenes. The hairy root culture did not produce shikonin on solid MS medium but produced the pigment in the root culture medium and also secreted it into the medium. Addition of absorbents; XAD-2, XAD-4, charcoal and so on increased the concentration of shikonin produced. The roots were cultivated in a 2 L air-lift type fermentor connected to a XAD-2 column (25 g) through a peristatic pump and 5 mg/day of shikonin was continuously produced during a period of more than 220 days.

Anthocyanins

Anthocyanins are the large group of water-soluble pigments responsible for many of the bright colours seen in flowers and fruit. They are composed of an aglycone (anthocyanidin) and more than one sugar moiety and normally change colour over the pH range due to the existence of four pH-dependent forms. Thus, at low pH they are red and at higher pH value (over 6) they turn blue. They are commonly used in acidic solutions in order to impart a red colour to soft drinks, sugar confectionary, jams and bakery toppings. The major source of anthocyanins for commercial purposes are grape pomaces and waste from juice and wine industries, but other potential sources have been investigated.

Crude preparations of anthocyanins are used extensively in the food industry and it has been claimed that pure anthocyanins are priced $1,250-2,000/kg, but crude materials are rather inexpensive.

Commercial exploration of cell cultures for anthocyanins therefore, has not been tackled seriously. Although there have been many papers describing the production of anthocyanins using cultured cells of various plant species, most of them seem to use an anthocyanin-producing cell line as a model system for secondary product production because of their colour which allows production to be easily visualized.

Among them, Yamamoto et al. of Nippon Paint Co. in Japan have studied production of anthocyanins intensively. They induced callus from Euphorbia millii leaves on MS medium containing 2,4-D, NAA, natural sources such as malt extract and yeast extract. As a major component in the callus, they identified cyanidin-3-arabinoside. The callus consisted of cell aggregates was cut to small pieces and cultivated on solid agar media. High producing cell aggregates were selected visually and they were transferred to fresh agar media. This procedure was repeated 28 times and one of the cells was determined to produce 1.32% d.w. anthocyanins in the cells. The levels of the pigments in flowers and leaves were 0.28% and less than 0.01%, respectively. They also established suspension cultures of E. millii.

Accumulation of anthocyanins was enhanced by a high osmotic potential in Vitis vinifera L. (grape) cell suspension cultures. They added sucrose or mannitol in the medium to increase the osmotic pressure and found the level of anthocyanins accumulated was increased to 1.5 times, 550 µg/10 cells.

Safflower Yellow

This yellow pigment obtained from the floret of the safflower plant (Carthamus tinctorius L.), is also known as Mexican saffron or American saffron, although it has no relation to genuine saffron. The major pigment is carthamin, which exists at levels of up to 30% in the flowers, and there is also a red pigment in concentrations of about 0.5%. Carthamin is the quinoid form of isocarthamin, the glucoside of 2',3',4',6'-tetrahydro-chalcone. Safflower yellow is not approved for use in the U.S. or in the E.E.C., but regulations do permit its use in Japan. It is stable to heat and light and is used in baked goods and beverages.

The production of carthamin from safflower callus cultures has been described by Kibun Co. in Japan. The callus was obtained from flower bud explants and could also be put into suspension. Medium optimization has been performed. The production of alpha-tocopherol (the tocopherol with the highest vitamin E activity) has been described for safflower cultures. Selection with various media components and precursor feeding experiments have enhanced the production. Kusaka et al.found that addition of cellulose, chitin or chitosan increased production of the red pigment. These polysaccharides appeared to show an eliciting activity. Addition of 1 mM D-phenylalanine and removal of Mg alone or both Mg and Ca from the culture medium also increased the production.

Plant cell cultures cannot presently be used for the production of these metabolites, given the high cost of the technology and the low value products, ie. $50-$80 and $48 for tocopherol and carthamin respectively.

Saffron

Saffron is the name of the spice which is made from the stamens of Crocus sativus and are prized for their use as a flavoring and colourant. The stigma of the plant contains crocin (yellow pigment), safranal (a fragrance) and picocrocin (bitter substance). The plant is grown mainly in Spain and India and it requires about 30,000-35,000 hand-picked blooms to produce 1 lb of dry saffron.

Crocin, being a glycoside, is water-soluble and is not soluble in oils and fats. Saffron is sensitive to pH changes and is unstable towards light and oxidative conditions, but it is moderately resistant to heat. It is used in baked goods, soups, meat and curry products, cheese, confectionary and as a condiment for the rice of Spanish and Indian foods. Saffron is also reputed to have medicinal value for stomach ailments.

The very high value of the product is due mainly to the fact that the life of the flowers is very short, making harvesting difficult. Thus, this is an ideal target for plant tissue cultures. Ajinomoto of Japan have approached this problem through the propagation of saffron stigma-like structures in vitro. Further studies showed that crocin and picrocrocin were present and, after heat treatment (as done with field-grown stigmas), safranal was produced. The composition of these phytochemicals corresponded with that of similarly-treated young, intact stigmas.

Madder Colorants

Rubia tinctorum (Rubiaceae) is a perennial plant, madder, originated from the coastal regions of the Mediterranean and its

roots have been used as red dyes in western Europe. The major components in the pigment are alizarin, purpurine and its glycoside, ruberythric acid. Pure alizarin is an orange crystal and is soluble at 1 part to 300 in boiling water and in other solvents. The R. tinctorum pigment, so-called madder colorant, shows a yellow color in acidic to neutral pH and tends to be reddish with increasing pH. It is highly resistant to heat and light which is favorable to food industry.

The callus of R. tinctorum was induced from the root of a germ-free plant by Odake et al.of San-Ei Chemical Industries in Japan and was grown on LS agar medium containing 2,4-D (10^{-5} M) and 0.2% gellan gum. Through the selection of high-producing cell lines and successive transfers, yellow pigment-producing cells were obtained which were then transferred into a liquid LS medium containing 10^{-6} M 2,4-D, 10^{-6} M kinetin and 3% sucrose. After 21 days cultivation in a 100 L jar fermentor, approximately 1.5 g of the pigment was extracted.

In order to remove auxins such as 2,4-D and IAA which are not desirable for food industry, a hairy root culture of R. tinctorum was established by the same group using Agrobacterium rhizogenes. They used a 5 mm diameter disc obtained from a leaf of R. tinctorum. It was incubated with cells of A. rhizogenes in suspension. After 78 hours at 25 0C in the dark, the leaf disc was transplanted to a hormone-free solid LS medium containing claforan (0.05%), sucrose (3%) and gellan gum (0.2%). Fourteen days later, hairy roots were found to produce the pigment. Using a 100 L bubble-column type jar fermentor, the hairy roots were cultivated for 21 days and approximately 800 mg of the pigment was obtained.

Miscellaneous

Chicle

Chicle is the most important raw material in chewing-gums and is made from the latex of Achras sapota Linn. Chicle contains approximately 60% of resin and 15% of rubber. The resin consists of lupeol, a-amyrin and ß-amyrin, and the rubber fraction contains cis- and trans-1,4-polyisoprene, however the biosynthetic pathway of chicle and its regulation mechanisms have not been elucidated although components of chicle are suggested to be synthesized through the mevalonic acid pathway.

Itoh et al. of Lotte Central Laboratory Co. Ltd., has tried to manufacture chicle by A. sapota tissue cultures. The callus induced from young shoots on a Linsmaier-Skoog's medium was shown to produce lupeol acetate, palmitate and stearate neither a,ß-amyrin nor rubber. On the other hand, the cells cultivated in suspension produced triterpenes as well as phytosterols such as campesterol, cholesterol, ß-sitosterol and stigmasterol.

In spite of many efforts for optimization of the culture conditions in suspension, the yield of triterpenes was not high enough for commercial application of the cell culture, therefore the researchers induced callus tissue of Dyera costulata and Couma macrocarpa, both of which were known to produce latexes for chicle as well. The callus tissues of both plants produced triterpenes at higher levels than those in the intact plants, respectively, but not the rubber.

Mucilage

Polysaccharides produced by Astragalas gummifer have been used as additives of ice cream and edible dressings.

Isa et al.of Q.P. Corp. in Japan used a hairy-root culture technology in order to establish an alternative method of production of mucilages of A. gummifer because of high cost and unstable supply of natural gums.

A. rhizogenes was inoculated in the stem of the in vitro plantlet of A. gummifer, which was incubated for approximately 14 days. Hairy root tips induced at the inoculated site were excised and cultivated on a hormone-free MS medium solidified with 0.2% Gelrite containing 500 mg/L Clafovan, a cefotaxim antibiotic. After successive transfers on the solid medium, the hairy roots were transferred into 30 ml of hormone-free liquid medium in a 100 ml Erlenmyer flask and cultivated at 25oC in the dark with constant agitation at 60 r.p.m.

The cells transformed by A. rhizogenes were found to produce opines as well as several types of water soluble mucilages. The composition of monosaccharides such as glucose, arabinose, galactose and xylose in the mucilages obtained from different hairy root lines varied widely, but the chemical structure of mucilages in the mother plant is much more complicated.

Hernandulcin

Hernandulcin is a sesquiterpene compound having strong sweet taste isolated from Lippia dulcia (Verbenaceae), but it has not yet been approved by FDA in the U.S.A.

Sauerwein and Shimomura induced hairy roots of this plant using A. rhizogenes. The roots cultivated in MS liquid medium supplemented with 2% sucrose under 16 hr/day light accumulated 0.25 mg/g-d.w.of hernandulcin. Addition of 0.2-10 mg/L chitosan into the medium increased its level up to 5 times. The axenic shoot culture of L. dulcia on MS solid medium containing 2% sucrose was shown to produce a high concentration of hernandulcin, 2.9%-d.w.

Conclusions

There are still a number of commercially important products which are being extracted from mass produced field-plants. In order to circumvent various problems caused from these processes as indicated in the Introduction, plant cell culture technology has been expected to be an efficient and useful tool.

For more than 30 years, many researchers have investigated plant cell cultures for production of a variety of phytochemicals; however, in spite of their many efforts only two products such as shikonins and ginseng cells are so far being manufactured commercially. The reasons why this technology has scarcely been applied in industry are; low yield of plant metabolites, unstable producing ability of cultured cells and their slow growth rate. Therefore, at present the plant cell culture is not a cost-effective technology. In particular, any process using plant cell cultures is not favorable if the desirable products can be easily manufactured by chemical- or microbial fermentation methods.

However, a variety of scientific strategies have been investigated for improving the production ability of cultured cells and

substantial progress has been made. Undoubtedly, some plant metabolites are likely to be manufactured through plant cell cultures. In addition to the approaches described in these Lessons, several research groups have paid an attention to using recombinant DNA technology as a tool for improvement of cultured cells although it is not an easy task in secondary metabolism.

To overcome barriers hindering industrial application of plant cell cultures, however, it is required not only to follow the existing approaches but also to conduct more fundamental research, including elucidation of biosynthetic pathways of many useful secondary metabolites in plants and of the regulation mechanisms in their biosyntheses. Collaboration with a number of researchers in other scientific fields is also very helpful.

Questions

1. Give a brief account of some plant virus inhibitor drugs obtained from plants.
2. Write short notes on:
   a. Shikonin compounds
   b. Food additives
   c. Mucilage and Chicle.

Note